

APPLICATION FOR PATENT

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Title: METHOD AND PHARMACEUTICAL COMPOSITION
FOR THE TREATMENT OF MULTIPLE SCLEROSIS

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FIELD AND BACKGROUND OF THE INVENTION

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The present invention relates to methods and pharmaceutical compositions effective in breaking-down immunological tolerance the CXC chemokine interferon gamma-inducible protein 10 (IP-10), resulting in the generation of self specific immunity to IP-10, for the treatment of diseases, such as autoimmune diseases, in which IP-10 plays a pivotal role in disease onset and/or progression, e.g., multiple sclerosis (MS) and other inflammatory autoimmune diseases such as rheumatoid arthritis. Thus, in one particular, the present invention relates to the induction of protective immunity against multiple sclerosis, so as to prevent or treat multiple sclerosis by DNA vaccines or by neutralizing antibodies directed to IP-10.

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Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the central nervous system (CNS) which, for many

years and for a variety of experimental protocols, serves as a model for the human disease, multiple sclerosis (MS), a chronic degenerative disease marked by patchy destruction of the myelin that surrounds and insulates nerve fibers and mild to severe neural and muscular impairments, since in both diseases circulating leukocytes penetrate the blood brain barrier and damage myelin resulting in impaired nerve conduction and paralysis (1, 2).

Molecular biology techniques were previously used to follow leukocyte trafficking to the site of inflammation at the CNS of EAE rats, and a model that characterizes this process as a sequential multi-step event was suggested (3).

At first, a very limited repertoire of T-cells, named "the primary influx" interact with their target antigen at the site of inflammation, leading to the activation of the blood brain barrier to express various adhesion molecules and thus to increase its permeability to circulating leukocytes (3, 4). Enhanced permeability of this barrier allows a non-selective influx of leukocytes, which are named "the secondary influx". This influx correlates with disease onset (3, 5). Subsequently, antigen specific autoimmune T-cells either become anergic or undergo programmed cell death (apoptosis) leading to a remission in disease

severity (6). Inhibition of the secondary influx, by either soluble peptide therapy or anti-adhesion molecule blockade effectively prevented, or even reversed, an ongoing disease even though the primary influx remained apparent at the site of inflammation (3-5, 7). Taken together these results emphasize the important role of the non-selective leukocyte influx to a site of inflammation.

During the course of EAE development, various proinflammatory cytokines and chemokines are produced at the site of inflammation (40, 53-55).

Chemokines are chemoattractants that mediate leukocyte attraction and recruitment at the site of inflammation. As such, they are likely to be key mediators in the recruitment of the secondary influx of leukocytes at an inflamed target organ. This has motivated researchers to use the novel technology of naked DNA vaccination (8-17) and explore the therapeutic potential of anti-chemokine immunotherapy in EAE.

Based on the positions of the first two cysteines, the chemokines can be divided into four highly conserved but distinct supergene families C-C, C-X-C, C and the newly discovered C-X3-C (18, 19, 36-38). The C-C family is primarily involved in the activation of endothelium and for

chemoattraction of T cells and monocytes to the site of inflammation (20-32).

The protective competence of anti-C-C chemokine based immunotherapy has been demonstrated by Karpus *et al.* who blocked EAE
 5 in mice by immunizing them with rabbit anti-mouse polyclonal antibodies against macrophage inflammatory protein-1 α (MIP-1 α) (33), and more recently by Gong *et al.* who used an antagonist of monocyte chemoattractant protein 1 (MCP-1) to inhibit arthritis in the MRL-lpr mouse model (34). In another study, Berman *et al.* used *in situ*
 10 hybridization to demonstrate the dominant expression of MCP-1 in rat EAE brain (35). Still more recently, Barnes *et al.* used anti-human RANTES antibody to ameliorate adjuvant induced arthritis in the Lewis rat (87).

The pivotal role of the proinflammatory cytokine tumor necrosis
 15 factor alpha (TNF- α) in EAE has also been well characterized. TNF- α is produced by activated T cells (mostly Th1) and macrophages, and its elevated expression at the site of inflammation occurs during the critical phase of disease (55), at the time when the 'secondary influx' of leukocytes is apparent (3). Except for a single recent study carried out in genetically

modified animals (56), all investigators agree that TNF- α contributes to the proinflammatory process in EAE and MS (57-71). Early studies have shown that IFN- γ and TNF- α together exhibit a synergistic effect on enhancing expression of adhesion molecules on endothelial cells (61), and on eliciting the inflammatory process, which can be reversed by either anti-adhesion molecule immunotherapy (4), or by blocking TNF- α (57-61). More recent studies have demonstrated that inhibition of TNF- α activity by either neutralizing antibodies, or soluble TNF receptor therapy, effectively prevent, or even reverse EAE (62, 64, 66-71). Overexpression of TNF- α at the CNS aggravated the disease (65), whereas genetically impaired expression of this gene inhibited disease development and progression (63).

A major disadvantage in treating chronic diseases with xenogenic neutralizing antibodies lies in their immunogenicity. This has motivated investigators to develop chimeric humanized antibodies (reviewed in 50), and monoclonal antibodies engineered with human Ig heavy and light chain yeast artificial chromosome (YAC) (51). However, following repeated immunization, these engineered antibodies do trigger an apparently allotypic response.

DNA vaccines represent a novel means of expressing antigens *in vivo* for the generation of both humoral and cellular immune responses (10, 14, 39, 41-43). This technology has proven successful in obtaining immunity not only to foreign antigens and tumors, but also to self antigens, such as a T cell receptor V genes (17) or autologous cytokines (42).

Recently, an alternative approach to generate anti chemokine/cytokine protective immunity was developed. WO 00/06203 teaches the use naked DNA vaccines expressing under the control of a viral promoter the C-C chemokines MCP-1, MIP-1 α , macrophage inflammatory protein-1 β (MIP-1 β), regulation on activation normal T expressed and secreted (RANTES) or the cytokine tumor necrosis factor alpha (TNF- α) and a repeated immunostimulatory sequence (i.e., CpG motif) that serves as a DNA adjuvant (15, 39, 88, 89), in the induction of protective immunity against multiple sclerosis.

Repeated administrations of such self cytokines encoding DNA vaccines tolerance to each relevant gene product is broken and immunological memory is established (90-96). These studies have also demonstrated that following the initiation of a T cell mediated autoimmune condition this memory is "turned on" to provide protective

immunity (90-96). Thus, administration of either TNF- α , MIP-1 α or MCP-1 DNA vaccines rendered high state of resistance against two different autoimmune diseases EAE and adjuvant induced arthritis (AA) that could be adoptively transferred by the neutralizing antibodies generated in response to each gene product. C-C chemokines were selected as candidates for DNA vaccination mostly because of their well established role in cell migration to a target organ (22, 23, 44-49). Since DNA vaccination elicits both cellular and humoral responses against products of a given construct (10, 14, 41-43), it is difficult to know which of these responses contributed more to the development of resistance. Nevertheless, elicitation of these antibodies was found to be dependent on the development of an autoimmune condition and regulated by the immune system in accordance with disease progression (90, 94). This, however, could provide the immune system of a patient with an autoimmune condition a powerful tool with which it can restrain its own harmful activities (94). Hence, a point of concern is that as an adjuvant the CpG may select Th1 cells that may potentially aggravate autoimmunity.

The role of the CXC chemokine interferon gamma-inducible protein 10 (IP-10) in the regulation of EAE or MS is yet unknown. Recent

studies demonstrate IP-10's ability to stimulates the directional migration of activated T cells, particularly Th1 cells (97-99), including of human T cell in SCID mice (100). Other studies demonstrate that IP-10 is transcribed at the CNS of MS patients, and NOD mice with developing type I diabetes (101-104).

While conceiving the present invention it was hypothesized that if IP-10 has a pivotal role in the development and/or progression of EAE or MS, than, it cab be used for the induction of protective immunity against multiple sclerosis, so as to prevent or treat multiple sclerosis. One advantage of using IP-10 for the induction of protective immunity lies in that downregulation of IP-10 via naked DNA vaccination would also reduce the chances of directional migration of activated T cells, particularly Th1 cells, that may potentially aggravate autoimmunity and which may be selected via the CpG motif adjuvant activity.

SUMMARY OF THE INVENTION

Interferon gamma-inducible protein 10 (IP-10) is a CXC chemokine that stimulates the directional migration of activated T cells, particularly Th1 cells. While reducing the present invention to practice, it was found

that administration of plasmid DNA encoding self IP-10 was efficient in breaking down immunological tolerance to IP-10, resulting in the generation of self specific immunity to IP-10. Despite the fact that the plasmid vaccine contained a repeated immunostimulatory sequence (i.e., a CpG motif) that serves as a DNA adjuvant and that is known to redirect Th1 polarization, the vaccine redirected the polarization of myelin basic protein specific T cells into Th2 and conferred the vaccinated rats a high state of resistance against experimental autoimmune encephalomyelitis (EAE), a T cell mediated autoimmune disease of the central nervous system (CNS) which traditionally serve as an in animal model system of multiple sclerosis (MS). The vaccine also suppressed disease when being administered after its active induction. Self specific antibodies to IP-10 developed in protected animals could inhibit leukocyte migration in a Boyden chamber, alter the *in vivo* Th1/Th2 balance of autoimmune T cells, and adoptively transfer disease suppression. EAE resistance was associated with an apparent alteration in the *in vivo* Th1/Th2 balance of autoimmune T cells towards Th2.

Thus, according to one aspect of the present invention there is provided a method of breaking-down an immunological tolerance to

interferon gamma-inducible protein 10 in a subject, the method comprising administering to, or expressing within, the subject an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to break-down the immunological tolerance to interferon gamma-inducible protein 10.

According to another aspect of the present invention there is provided a method of breaking-down an immunological tolerance to interferon gamma-inducible protein 10 in a subject, the method comprising administering to the subject anti-interferon gamma-inducible protein 10 antibodies in an amount sufficient to break-down the immunological tolerance to interferon gamma-inducible protein 10.

According to still another aspect of the present invention there is provided a method of breaking-down an immunological tolerance to interferon gamma-inducible protein 10 in a subject, the method comprising directly or indirectly introducing anti-interferon gamma-inducible protein 10 antibodies to the subject in an amount sufficient to break-down the immunological tolerance to interferon gamma-inducible protein 10.

According to yet another aspect of the present invention there is provided a pharmaceutical composition for breaking-down an immunological tolerance to interferon gamma-inducible protein 10 in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable carrier approved for medical or veterinary administration and, as an active ingredient, an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, or of an expression construct encoding the interferon gamma-inducible protein 10, or the immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to break-down the immunological tolerance to interferon gamma-inducible protein 10.

According to an additional aspect of the present invention there is provided a pharmaceutical composition for breaking-down an immunological tolerance to interferon gamma-inducible protein 10 in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable carrier approved for medical or veterinary administration and, as an active ingredient, anti-interferon gamma-inducible protein 10 antibodies in an amount sufficient to break-down the immunological tolerance to interferon gamma-inducible protein 10.

According to yet another aspect of the present invention there is provided a method of generating self specific immunity to interferon gamma-inducible protein 10 in a subject, the method comprising administering to, or expressing within, the subject an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to generate self specific immunity to interferon gamma-inducible protein 10.

According to a further aspect of the present invention there is provided a method of generating specific immunity to interferon gamma-inducible protein 10 in a subject, the method comprising administering to the subject anti-interferon gamma-inducible protein 10 antibodies in an amount sufficient to generate self specific immunity to interferon gamma-inducible protein 10.

According to still a further aspect of the present invention there is provided a method of generating specific immunity to interferon gamma-inducible protein 10 in a subject, the method comprising directly or indirectly introducing anti-interferon gamma-inducible protein 10

antibodies to the subject in an amount sufficient to generate self specific immunity to interferon gamma-inducible protein 10.

According to yet a further aspect of the present invention there is provided a pharmaceutical composition for generating self specific immunity to interferon gamma-inducible protein 10 in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable carrier approved for medical or veterinary administration and, as an active ingredient, an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, or of an expression construct encoding the interferon gamma-inducible protein 10, or the immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to generate self specific immunity to interferon gamma-inducible protein 10.

According to another aspect of the present invention there is provided a pharmaceutical composition for generating self specific immunity to interferon gamma-inducible protein 10 in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable carrier approved for medical or veterinary administration and, as an active ingredient, anti-interferon gamma-inducible protein 10 antibodies in an

amount sufficient to generate self specific immunity to interferon gamma-inducible protein 10.

According to an additional aspect of the present invention there is provided a method of preventing or treating an autoimmune disease in which activity of interferon gamma-inducible protein 10 is pivotal in a subject, the method comprising administering to, or expressing within, the subject an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to treat or prevent the autoimmune disease.

According to still an additional aspect of the present invention there is provided a method of preventing or treating an autoimmune disease in which activity of interferon gamma-inducible protein 10 is pivotal in a subject, the method comprising administering to the subject anti-interferon gamma-inducible protein 10 antibodies in an amount sufficient to treat or prevent the autoimmune disease.

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subject, the method comprising directly or indirectly introducing anti-interferon gamma-inducible protein 10 antibodies to the subject in an amount sufficient to treat or prevent the autoimmune disease.

According to yet an additional aspect of the present invention there is provided a pharmaceutical composition for preventing or treating an autoimmune disease in which activity of interferon gamma-inducible protein 10 is pivotal in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable carrier approved for medical or veterinary administration and, as an active ingredient, an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, or of an expression construct encoding the interferon gamma-inducible protein 10, or the immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to treat or prevent the autoimmune disease.

According to still another aspect of the present invention there is provided a pharmaceutical composition for preventing or treating an autoimmune disease in which activity of interferon gamma-inducible protein 10 is pivotal in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable carrier approved for medical or

veterinary administration and, as an active ingredient, anti-interferon gamma-inducible protein 10 antibodies in an amount sufficient to treat or prevent the autoimmune disease.

According to a further aspect of the present invention there is
5 provided a method of restricting a polarization of myelin basic protein specific T cells into Th2 cells in a subject, the method comprising administering to, or expressing within, the subject an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10
10 antibodies so as to restrict a polarization of myelin basic protein specific T cells into Th2 cells.

According to still a further aspect of the present invention there is provided a method of restricting a polarization of myelin basic protein specific T cells into Th2 cells in a subject, the method comprising
15 administering to the subject anti-interferon gamma-inducible protein 10 antibodies in an amount sufficient to restrict a polarization of myelin basic protein specific T cells into Th2 cells.

According to yet a further aspect of the present invention there is provided a method of restricting a polarization of myelin basic protein

specific T cells into Th2 cells in a subject, the method comprising directly or indirectly introducing anti-interferon gamma-inducible protein 10 antibodies to the subject in an amount sufficient to restrict a polarization of myelin basic protein specific T cells into Th2 cells.

5 According to a further aspect of the present invention there is provided a pharmaceutical composition for restricting a polarization of myelin basic protein specific T cells into Th2 cells in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable carrier approved for medical or veterinary administration and, as an active
10 ingredient, an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, or of an expression construct encoding the interferon gamma-inducible protein 10, or the immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to restrict a polarization of myelin basic protein
15 specific T cells into Th2 cells.

 According to still another aspect of the present invention there is provided a pharmaceutical composition for restricting a polarization of myelin basic protein specific T cells into Th2 cells in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable

carrier approved for medical or veterinary administration and, as an active ingredient, anti-interferon gamma-inducible protein 10 antibodies in an amount sufficient to restrict a polarization of myelin basic protein specific T cells into Th2 cells.

5 According to yet a further aspect of the present invention there is provided a method of inducing protective immunity against multiple sclerosis in a subject, the method comprising administering to, or expressing within, the subject an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit
10 sufficient anti-interferon gamma-inducible protein 10 antibodies, so as to induce protective immunity against multiple sclerosis in the subject.

 According to another aspect of the present invention there is provided a method of inducing protective immunity against multiple sclerosis in a subject, the method comprising administering to the subject
15 anti-interferon gamma-inducible protein 10 antibodies in an amount sufficient to induce protective immunity against multiple sclerosis in the subject.

 According to still another aspect of the present invention there is provided a method of inducing protective immunity against multiple

sclerosis in a subject, the method comprising directly or indirectly introducing anti-interferon gamma-inducible protein 10 antibodies to the subject in an amount sufficient to induce protective immunity against multiple sclerosis in the subject.

5 According to still a further aspect of the present invention there is provided a pharmaceutical composition for inducing protective immunity against multiple sclerosis in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable carrier approved for medical or veterinary administration and, as an active ingredient, an amount of
10 interferon gamma-inducible protein 10, or an immunological portion thereof, or of an expression construct encoding the interferon gamma-inducible protein 10, or the immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies, so as to induce protective immunity against multiple sclerosis
15 in the subject.

According to yet another aspect of the present invention there is provided a pharmaceutical composition for inducing protective immunity against multiple sclerosis in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable carrier approved for medical or

veterinary administration and, as an active ingredient, anti-interferon gamma-inducible protein 10 antibodies in an amount sufficient to induce protective immunity against multiple sclerosis in the subject.

According to another aspect of the present invention there is
5 provided a method of preventing or treating multiple sclerosis in a subject, the method comprising administering to, or expressing within, the subject an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to treat or prevent multiple
10 sclerosis.

According to still another aspect of the present invention there is
provided a method of preventing or treating multiple sclerosis in a subject, the method comprising administering to the subject anti-interferon gamma-inducible protein 10 antibodies in an amount sufficient to treat or
15 prevent multiple sclerosis.

According to yet another aspect of the present invention there is provided a method of preventing or treating multiple sclerosis in a subject, the method comprising directly or indirectly introducing anti-interferon

gamma-inducible protein 10 antibodies to the subject in an amount sufficient to treat or prevent multiple sclerosis.

According to a further aspect of the present invention there is provided a pharmaceutical composition for preventing or treating multiple sclerosis in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable carrier approved for medical or veterinary administration and, as an active ingredient, an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, or of an expression construct encoding the interferon gamma-inducible protein 10, or the immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to treat or prevent multiple sclerosis.

According to still a further aspect of the present invention there is provided a pharmaceutical composition for preventing or treating multiple sclerosis in a subject, the pharmaceutical composition comprising, a pharmaceutically acceptable carrier approved for medical or veterinary administration and, as an active ingredient, anti-interferon gamma-inducible protein 10 antibodies in an amount sufficient to treat or prevent multiple sclerosis.

According to further features in preferred embodiments of the invention described below, expressing within the subject the amount of interferon gamma-inducible protein 10 is by generating in, or introducing into, the subject cells expressing recombinant interferon gamma-inducible
5 protein 10, or an immunological portion thereof.

According to still further features in the described preferred embodiments generating in the subject cells expressing recombinant interferon gamma-inducible protein 10, or an immunological portion thereof, is by vaccinating the subject with an expression construct
10 encoding interferon gamma-inducible protein 10, or the immunological portion thereof.

According to still further features in the described preferred embodiments the pharmaceutical composition is packaged and identified for treatment of a disease or condition in which interferon
15 gamma-inducible protein 10 plays a pivotal role.

The present invention successfully addresses the shortcomings of the presently known configurations by providing novel methods and compositions with which to combat autoimmune diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1A-D demonstrate that IP-10 encoding DNA vaccine redirect antigen specific T cell polarization and suppresses EAE. A group of nine Lewis rats was subjected to four weekly injections of naked DNA encoding IP-10. Control rats (nine per group) were either injected with the pcDNA3 vector alone, or with PBS. Two months after the last

immunization all rats were immunized with p68-86/CFA to induce active EAE. Just before the onset of disease (day 9) three rats per groups were sacrificed and spleen T cells were cultured together with MBPp68-86 for cytokine determination. The remaining six rats were monitored for the development of active EAE by an observer blind to the experimental protocol. Figure 1A shows mean maximal score \pm SE of these groups. Figures 1B-D show the levels of TNF- α , IL-4 and IFN- γ that was determined (mean triplicates \pm SE) in supernatants of spleen cells cultured as described above.

FIGs. 2A-C demonstrate that IP-10 encoding DNA vaccine induces breakdown of tolerance to its gene product and generates immunity to native IP-10. Groups of Lewis rats were subjected to a repeated administration of IP-10 encoding DNA vaccines and then to active induction of EAE as described in the legend to Figure 1. An additional group was subjected to IP-10 encoding DNA vaccine and later on immunized with CFA alone to induce a local inflammatory response. At different time points blood serum was obtained and evaluated for IP-10 specific antibody titer. On day 12 (peak of disease in control rats) SCF was also obtained and subjected to the same evaluation. Figure 2A shows by

Western Blot demonstrating that the self specific anti IP-10 antibodies bind to recombinant rat IP-10 (10 kDa) and also to the commercially available mouse IP-10 (8.7 kDa fragment, Cytolab, Rehovot, Israel). These antibodies also bound natural rat IP-10 from supernatant of activated MBP p68-86 specific cultured T cells (not shown). Figure 2B shows the IP-10 specific antibody titer developed in blood serum and SCF of representative rats from each of the above groups (Mean antibody titer obtained from 3 rats per group \pm SE). Figure 2C shows the kinetics of antibody titer to self IP-10 in pcDNA3-IP vaccinated rats that were subjected to either active induction of EAE or administration of CFA alone (Mean antibody titer obtained from 3 rats per group \pm SE).

FIGs. 3A-C demonstrate that IP-10-specific antibodies generated in DNA vaccinated EAE rats are neutralizing antibodies that inhibit both migratory properties (Figure 3A) and polarization of activated T cells, as was measured by direct ELISA (Figure 3B) or by intracellular FACS analysis (Figure 3C). In Figure 3A, IP-10 specific antibodies (IgG, CNBr purified) were tested for their ability to inhibit the migration of an encephalitogenic cell line (L68-86) in a Boyden chemotaxis chamber assay. Rat IP-10 and the 8.7 kDa fraction of mouse IP-10 (Cytolab), each

at a concentration of 200 ng/ml, were used as chemoattractants. fMLP (Sigma) at a concentration of 10^{-7} M was used as a positive control for chemoattraction. Result are shown as mean of triplicates \pm SE. Boyden chemotaxis chamber assay. The experimental protocol for Figures 3B and 3C is as follows: Nine days after active EAE induction primary spleen T cells were cultured with 40 μ g/ml of MBP p68-86 with or without, the addition of 10 ng/ml of either anti IP-10 antibodies, antibodies from control AE rats vaccinated with an empty plasmid, control IgG from naïve rats or medium. Production of TNF- α (Figure 3A), IFN- γ (Figure 3B) and IL-4 (Figure 3C) were determined 72 h later. At that time CD4⁺ spleen T cells (W3/25⁺) from cultures that were or were not supplemented with anti IP-10 antibodies were subjected to intracellular FACS analysis of IFN- γ vs IL-4 (Figure 3C).

FIGS. 4A-D demonstrate that administration of self specific antibodies to IP-10 redirects antigen specific T cell polarization towards Th2 and suppresses EAE. Four groups of nine rats were subjected to active induction of EAE. During the first five days following disease induction these rats were repeatedly (every other day) subjected to injections of 100 μ g/rat of either anti IP-10 antibodies, IgG from pcDNA3

vaccinated EAE rats or from normal rat serum. Another control group was administered with PBS. Three rats per group were sacrificed on day 10 and their spleen T cells were cultured together with MBPp68-86 for cytokine determination (Figures 4B-D) and FACS analysis (Figure 5). The remaining rats were daily monitored for clinical signs of disease by an observer blind to the experimental protocol. Figure 4A shows mean maximal score \pm SE of these groups. Figures 4B-D show the levels of TNF- α , IL-4 and IFN- γ that were determined (mean triplicates \pm SE) in supernatants of spleen cells derived from the rats and cultured as described above.

FIGS. 5A-B demonstrate intracellular staining of IL-4 and IFN- γ in primary spleen cell cultures of rats administered with anti-IP-10 specific antibodies. Cultured spleen cells (48 hours of *in vivo* stimulation) derived from EAE rats that were subjected to treatment with anti-IP-10 specific antibodies (Figure 5A), PBS (Figure 5B), or IgG from normal rat serum (not shown), were analyzed for intracellular IL-4 vs. IFN- γ of CD4+ (w3/25+) T cells. No difference were recorded between T cells from rats which received no treatment (Figure 5A) to those from rats treated with normal rat IgG (not shown).

FIG. 6 demonstrate that IP-10 encoding DNA vaccines interfere in the regulation of established EAE. Lewis rats (6 per group) were immunized with MBP₆₈₋₈₆/CFA to induce active EAE, five, six and seven days later injected with either IP-10 or soluble β -actin encoding DNA vaccines (300 μ g/rat per injection) and monitored for the development and progression of disease by an observer blind to the experimental procedure. The results are shown as mean maximal score \pm SE. Sixteen days after disease induction blood sera were obtained from 3 representative rats per group and determined for anti-IP-10 and anti- β actin specific antibody titers. Results are shown as mean log₂ antibody titer of 3 samples \pm SE.

FIG. 7 demonstrates that demonstrate that IP-10 encoding DNA vaccines can be used to treat established EAE. C57/BL mice were subjected to active induction of EAE. On day 15 these mice were separated into four groups of equally sick mice (six mice per group) and subjected to a repeated administration (3 times, days 15, 16, 17) of either IP-10 or soluble β -actin encoding DNA vaccines, empty vector (100 μ g each) or PBS. The results are shown as mean maximal score \pm SE.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of methods and pharmaceutical compositions which can be used for breaking-down immunological tolerance the CXC chemokine interferon gamma-inducible protein 10 (IP-10), resulting in the generation of self specific immunity to IP-10, for the treatment of diseases, such as autoimmune diseases, in which IP-10 plays a pivotal role in disease onset and/or progression. Specifically, the present invention can be used to induce protective immunity against multiple sclerosis, so as to prevent or treat multiple sclerosis.

The principles and operation of the methods and compositions according to the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

While reducing the present invention to practice, as is described in detail in the Examples section that follows, a significant anti-self response to IP-10 in rats immunized with MBPp68-86/CFA to induce active EAE, but not with CFA alone to induce a local inflammatory response was observed. This response accelerates in EAE rats following DNA vaccination to provide protective immunity. This type of intervention was turned on rapidly and could effectively suppress an established disease.

The mechanistic basis of the adjuvant effect of naked DNA vaccines with CpG motif has been solved very recently (88, 89). Either by activating Toll-like receptor 9 (TLR9) (88), and/or via the catalytic subunit of DNA-PKcs (89), a CpG motif initiates a signal transduction cascade in antigen presenting cells (e.g., dendritic cells) resulting in their activation. An abundant use of naked DNA vaccines with plasmids barring this motif is for the increase of the pro-inflammatory (Th1) immune response against infectious agents such as tuberculosis, HIV and allergens such as mite proteins (15, 39, 106-110). Interestingly, repeated administrations of DNA vaccines that include the CpG motif did not ameliorate T cell mediated autoimmunity in some previous experiments (90, 92-96, 17, 111). A recent observations suggest that CpG selects *in*

vivo a low TNF- α producing subtype of CD4⁺ “Th1” cells (S. Youssef, G. Wildbaum and N. Karin, In preparation). This can explain, in part, why “pro-Th1” DNA vaccines do not aggravate autoimmunity. Hence, it is demonstrated herein, for the first time, how a CpG containing DNA vaccine could be constructed to preferentially select high IL-4, low IFN- γ , low TNF- α producing T cells. Such a construct could be used alone or together with a construct encoding a self autoimmune target antigen (such as MBP, PLP or MOG for MS) to effectively direct regulatory T cells to an autoimmune site.

Recent studies demonstrated the ability of IP-10 to stimulates the directional migration of activated T cells, particularly Th1 cells (97-99). Neutralizing the *in vivo* activity of IP-10 could affect T cell balance either because less Th1 cell are accumulated at the autoimmune site and/or because IP-10 is directly involved in T cell polarization. It is still an opened question, however, whether IP-10 may shift the Th1 Th2 balance towards Th2 as a result of a direct effect on T cell polarization. The data presented herein, showing that anti-IP-10 specific antibodies alter the *in vivo* balance towards high IL-4, low IFN- γ producing T cells (44 % vs 18 %) indicates that IP-10 is a chemokine with a dual function. Interestingly,

it was previously shown that neutralizing antibodies to IL-18 not only can direct the *in vivo* polarization of autoimmune T cell and thus suppress EAE, but also the *in vivo* polarization of primary T cells (105).

Thus, according to one aspect of the present invention there is provided a method of breaking-down an immunological tolerance to interferon gamma-inducible protein 10 in a subject. The method is effected by administering to, or expressing within, the subject an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to break-down the immunological tolerance to interferon gamma-inducible protein 10. Alternatively, the method is effected by direct administration of anti-interferon gamma-inducible protein 10 antibodies so as to break-down the immunological tolerance to interferon gamma-inducible protein 10.

As used herein, the phrase "breaking-down an immunological tolerance" refers to generating self specific immunity against a self component.

As used herein, the term "subject" refers to an animal having an immune system, preferably a mammal, such as a human being, household pets, farm animals and mammals held in captivity.

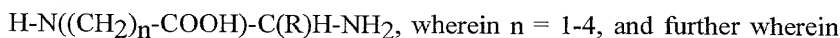
Hence, according to another aspect of the present invention there is
5 provided a method of generating self specific immunity to interferon gamma-inducible protein 10 in a subject. The method is effected by administering to, or expressing within, the subject an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10
10 antibodies so as to generate self specific immunity to interferon gamma-inducible protein 10. Alternatively, the method is effected by directly administering anti-interferon gamma-inducible protein 10 antibodies so as to generate self specific immunity to interferon gamma-inducible protein 10.

15 As used herein, the phrase "interferon gamma-inducible protein 10, or an immunological portion thereof" refers to the entire interferon gamma-inducible protein 10 protein and also to a peptide portion thereof which includes at least one continuous or discontinuous immunogenic epitope.

As used herein the term "peptide" includes native peptides (either degradation products, synthetically synthesized peptides or recombinant peptides) and peptidomimetics (typically, synthetically synthesized peptides), such as peptoids and semipeptoids which are peptide analogs, which may have, for example, modifications rendering the peptides more stable while in a body, or more immunogenic. Such modifications include, but are not limited to, cyclization, N terminus modification, C terminus modification, peptide bond modification, including, but not limited to, $\text{CH}_2\text{-NH}$, $\text{CH}_2\text{-S}$, $\text{CH}_2\text{-S=O}$, O=C-NH , $\text{CH}_2\text{-O}$, $\text{CH}_2\text{-CH}_2$, S=C-NH , CH=CH or CF=CH , backbone modification and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified, for example, in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein. Further detail in this respect are provided hereunder.

Thus, a peptide according to the present invention can be a cyclic peptide. Cyclization can be obtained, for example, through amide bond formation, e.g., by incorporating Glu, Asp, Lys, Orn, di-amino butyric (Dab) acid, di-aminopropionic (Dap) acid at various positions in the chain

(-CO-NH or -NH-CO bonds). Backbone to backbone cyclization can also be obtained through incorporation of modified amino acids of the formulas



- 5 R is any natural or non-natural side chain of an amino acid.

Cyclization via formation of S-S bonds through incorporation of two Cys residues is also possible. Additional side-chain to side chain cyclization can be obtained via formation of an interaction bond of the formula $\text{-(CH}_2\text{)}_n\text{-S-CH}_2\text{-C-}$, wherein $n = 1$ or 2 , which is possible, for
 10 example, through incorporation of Cys or homoCys and reaction of its free SH group with, e.g., bromoacetylated Lys, Orn, Dab or Dap.

- Peptide bonds (-CO-NH-) within the peptide may be substituted, for example, by N-methylated bonds (-N(CH₃)-CO-), ester bonds (-C(R)H-C-O-O-C(R)-N-), ketomethylen bonds (-CO-CH₂-), o-aza bonds
 15 (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH₂-NH-), hydroxyethylene bonds (-CH(OH)-CH₂-), thioamide bonds (-CS-NH-), olefinic double bonds (-CH=CH-), retro amide bonds (-NH-CO-), peptide derivatives (-N(R)-CH₂-CO-), wherein R is the "normal" side chain, naturally presented on the carbon atom.

These modifications can occur at any of the bonds along the peptide chain and even at several (2-3) at the same time.

Natural aromatic amino acids, Trp, Tyr and Phe, may be substituted for synthetic non-natural acid such as TIC, naphthylelanine (Nol),
 5 ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr.

Tables 1-2 below list all the naturally occurring amino acids (Table 1) and non-conventional or modified amino acids (Table 2).

TABLE 1

Amino Acid	Three-Letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F

Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

TABLE 2

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
α -amino- α -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
aminocyclopropane-	Cpro	L-N-methylasparagine	Nmasn
carboxylate		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-	Norb	L-N-methylglutamine	Nmgin
carboxylate		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisoleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Das	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Diie	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr

D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib
D-valine	Dval	α -methyl- γ -aminobutyrate	Mgabu
D-O-methylalanine	Dmala	α -methylcyclohexylalanine	Mchexa
D-O-methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpen
D-O-methylasparagine	Dmasn	α -methyl-O-naphthylalanine	Manap
D-O-methylaspartate	Dmasp	α -methylpenicillamine	Mpen
D-O-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D-O-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D-O-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-O-methylisoleucine	Dmile	N-amino-O-methylbutyrate	Nmaabu
D-O-methylleucine	Dmleu	α -naphthylalanine	Anap
D-O-methyllysine	Dmlys	N-benzylglycine	Nphe
D-O-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-O-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-O-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-O-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-O-methylserine	Dmser	N-cyclobutylglycine	Nebut
D-O-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D-O-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D-O-methyltyrosine	Dmty	N-cyclodecylglycine	Ndec
D-O-methylvaline	Dmval	N-cyclododecylglycine	Ndod
D-O-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-O-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro

D-O-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-O-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-O-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl) glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmt
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpn
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nva
D-N-methyltyrosine	Dnmtyr	N-methyl- α -naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-O-methylalanine	Mala
L-O-methylarginine	Marg	L-O-methylasparagine	Masn
L-O-methylaspartate	Masp	L-O-methyl- <i>t</i> -butylglycine	Mtbug
L-O-methylcysteine	Mcys	L-methylethylglycine	Metg
L-O-methylglutamine	Mgln	L-O-methylglutamate	Mglu
L-O-methylhistidine	Mhis	L-O-methylhomo phenylalanine	Mhphe
L-O-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp

D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmt
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyl- α -naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- α -methylalanine	Mala
L- α -methylarginine	Marg	L- α -methylasparagine	Masn
L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
L- α -methylhistidine	Mhis	L- α -methylhomophenylalanine	Mhphe
L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L- α -methylleucine	Mleu	L- α -methyllysine	Mlys
L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
L- α -methylserine	mser	L- α -methylthreonine	Mthr
L- α -methylvaline	Mtrp	L- α -methyltyrosine	Mtyr
L- α -methylleucine	Mval Nnbhm	L-N-methylhomophenylalanine	Nmhph
N-(N-(2,2-diphenylethyl)		N-(N-(3,3-diphenylpropyl)	

carbamylmethyl-glycine	Nnbhm	carbamylmethyl(1)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl ethylamino)cyclopropane	Nmbc		

A peptide according to the present invention can be used in a self standing form or be a part of moieties such as proteins and display moieties such as display bacteria and phages.

5 Additionally, a peptide according to the present invention includes at least five, optionally at least six, optionally at least seven, optionally at least eight, optionally at least nine, optionally at least ten, optionally at least eleven, optionally at least twelve, optionally at least thirteen, optionally at least fourteen, optionally at least fifteen, optionally at least
10 sixteen or optionally at least seventeen, optionally between seventeen and twenty five or optionally between twenty five and at least thirty amino acid residues (also referred to herein interchangeably as amino acids).

Accordingly, as used herein in the specification and in the claims section below the term "amino acid" or "amino acids" is understood to
15 include the 20 naturally occurring amino acids; those amino acids often modified post-translationally *in vivo*, including, for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acids including, but not limited to, 2-aminoadipic acid,

hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine.

Furthermore, the term "amino acid" includes both D- and L-amino acids.

The peptides of the invention can be derived from a specified protein or proteins and further from homologous regions of proteins
5 homologous to the specified proteins of the same or other species, provided that these peptides are therapeutically effective. The term further relates to permissible amino acid alterations and peptidomimetics designed based on the amino acid sequence of the specified proteins or their homologous proteins.

10 As used herein the term "epitope" refer to a region of a molecule, such as, for example, the peptide(s) of the present invention, which region is characterized by specific molecular arrangement so as to elicit an immunological response thereto. When derived from a molecule which is linear by nature, yet acquires a complex three dimensional structure in
15 which regions which are distant from one another in the linear topography are close to one another in the complex three dimensional structure, such as a protein, an epitope can either be continuous, i.e., defined by a contiguous sequence, or discontinuous, i.e., defined by a combination of at least two non-contiguous regions of the sequence.

According to an additional aspect of the present invention there is provided a method of preventing or treating an autoimmune disease in which activity of interferon gamma-inducible protein 10 is pivotal in a subject, the method comprising administering to, or expressing within, the
5 subject an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to treat or prevent the autoimmune disease. Alternatively, the method comprising anti-interferon gamma-inducible protein 10 antibodies so as to treat or prevent the
10 autoimmune disease.

As used herein, the term "treat" includes substantially inhibiting, slowing or reversing the progression of a disease, substantially ameliorating clinical symptoms of a disease or substantially preventing the appearance of clinical symptoms of a disease.

15 There are several autoimmune diseases in which activity of interferon gamma-inducible protein 10 is pivotal. These include, for example, multiple sclerosis, the pivotality of interferon gamma-inducible protein 10 in its development and progression is exemplified herein. Worth mentioning in this respect is that IP-10 was found to be highly

expressed in demyelinating brain lesions of multiple sclerosis patients (113), the insulin producing beta islands of diabetic NOD mice (102), liver biopsies of patients suffering from chronic hepatitis (114), and its chemokine receptors CXCR3 is highly expressed in Immunostaining of T
5 cells in rheumatoid arthritis synovial fluid (115).

Thus, according to still an additional aspect of the present invention there is provided a method of restricting a polarization of myelin basic protein specific T cells into Th2 cells in a subject, the method comprising administering to, or expressing within, the subject an amount of interferon
10 gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to restrict a polarization of myelin basic protein specific T cells into Th2 cells. Alternatively, the method comprising directly
15 administering anti-interferon gamma-inducible protein 10 antibodies so as to restrict a polarization of myelin basic protein specific T cells into Th2 cells.

According to a specific embodiment, the present invention provides a method of inducing protective immunity against multiple sclerosis in a subject. This method is effected by administering to, or expressing within,

the subject an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies, so as to induce protective immunity against multiple sclerosis in the subject. Alternatively, the method is effected by directly administering anti-interferon gamma-inducible protein 10 antibodies, so as to induce protective immunity against multiple sclerosis in the subject.

According to still another aspect of the present invention there is provided a method of preventing or treating multiple sclerosis in a subject, the method comprising administering to, or expressing within, the subject an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies so as to treat or prevent multiple sclerosis. Alternatively, the method comprising directly administering anti-interferon gamma-inducible protein 10 antibodies so as to treat or prevent multiple sclerosis.

In one of its embodiments, the present invention is practiced by administration of anti IP-antibodies to a subject in need. In the Examples section that follows anti-IP-10 antibodies elicited via DNA vaccination

were affinity purified and administered to EAE rats, resulting in prevention of disease development and/or progression.

As used herein in the specification and in the claims section below, the term "antibody" refers to any monoclonal or polyclonal immunoglobulin, or a fragment of an immunoglobulin such as sFv (single chain antigen binding protein), Fab1 or Fab2. The immunoglobulin could also be a "humanized" antibody, in which antibody variable regions of an animal (e.g., murine) are fused to human constant regions, or in which complementarity-determining regions are grafted onto a human antibody structure (Wilder, R.B. et al., J. Clin. Oncol., 14:1383-1400, 1996). Unlike mouse or rabbit antibodies, "humanized" antibodies often do not undergo an undesirable reaction with the immune system of the subject. The terms "sFv" and "single chain antigen binding protein" refer to a type of a fragment of an immunoglobulin, an example of which is sFv CC49 (Larson, S.M. et al., Cancer, 80:2458-68, 1997).

The elicitation and subsequent production of antibodies according to the present invention is through *in vivo* or *in vitro* techniques, the antibody having been prepared by a process comprising the steps of (a) exposing cells capable of producing antibodies to the IP-10 protein or the

immunological portion thereof and thereby generating antibody producing cells; (b) immortalizing the antibody producing cells by viral transformation of by fusing the antibody producing cells with myeloma cells and thereby generating a plurality of immortalized cells each producing monoclonal antibodies; and (c) screening the plurality of monoclonal antibodies to identify a monoclonal antibody which specifically binds IP-10.

A clone that produces high amounts of efficient anti-IP-10 monoclonal antibody is then propagated and used to produce large amounts of the antibody. The antibody is preferably affinity purified against the IP-10 protein. The genes encoding the antibody can be clones and manipulated using techniques well known in the art so as to generate the single chain and/or humanized antibodies.

Gene therapy as used herein refers to the transfer of genetic material (e.g., DNA or RNA) of interest into a host to treat or prevent an acquired disease or condition or phenotype. The genetic material of interest encodes a protein product whose production *in vivo* is desired. For review see, in general, the text "Gene Therapy" (Advanced in Pharmacology 40, Academic Press, 1997).

Two basic approaches to gene therapy have evolved: (i) *ex vivo* and (ii) *in vivo* gene therapy. In *ex vivo* gene therapy cells are removed from a patient, and while being cultured are treated *in vivo*. Generally, a functional sequence is introduced into the cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the host/patient. These genetically reimplanted cells have been shown to express the transfected genetic material *in situ*.

In *in vivo* gene therapy, target cells are not removed from the subject rather the genetic material to be transferred is introduced into the cells of the recipient organism *in situ*, that is within the recipient. These genetically altered cells have been shown to express the transfected genetic material *in situ*.

The gene expression construct is capable of delivery/transfer of heterologous nucleic acid into a host cell. The expression construct may include elements to control targeting, expression and transcription of the nucleic acid in a cell selective manner as is known in the art. It should be noted that often the 5'UTR and/or 3'UTR of the gene may be replaced by

the 5'UTR and/or 3'UTR of the expression construct. Therefore, as used herein the expression construct may, as needed, not include the 5'UTR and/or 3'UTR of the actual gene to be transferred and only include the specific amino acid coding region.

5 The expression construct can include a promoter for controlling transcription of the heterologous material and can be either a constitutive or inducible promoter to allow selective transcription. Enhancers that may be required to obtain necessary transcription levels can optionally be included. Enhancers are generally any nontranslated DNA sequence
10 which works contiguously with the coding sequence (in *cis*) to change the basal transcription level dictated by the promoter. The expression construct can also include a selection gene as described herein below.

Expression constructs can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be
15 found generally described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York 1989, 1992), in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Maryland 1989), Chang *et al.*, Somatic Gene Therapy, CRC Press, Ann Arbor, MI 1995), Vega *et al.*, Gene Targeting,

CRC Press, Ann Arbor MI (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston MA 1988) and Gilboa *et al.* (Biotechniques 4 (6): 504-512, 1986) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see United States patent 4,866,042 for vectors involving the central nervous system and also United States patents 5,464,764 and 5,487,992 for positive-negative selection methods.

In some cases, introduction of nucleic acids by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

A specific example of DNA viral vector introducing and expressing recumbent sequences is the adenovirus-derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired

recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells and can include, for example, an *in vivo* or *ex vivo* culture of cells, a tissue or a human subject.

Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

In addition, recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be

useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection.

- 5 In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's
- 10 genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot
- 15 proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

The recombinant vector can be administered in several ways. If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration. Following injection, the viral vectors will circulate until they recognize host cells with appropriate target specificity for infection.

Although, the most common problems encountered in prior art gene therapy protocols are poor efficacy and immune response of the host to the vector, these problems are of lesser or no influence while practicing the present invention as following a brief period of expression immunological memory develops.

Thus, according to the present invention, expressing within the subject the amount of interferon gamma-inducible protein 10 can be effected by generating in (*in vivo* gene therapy), or introducing into (*ex*

vivo gene therapy), the subject cells expressing recombinant interferon gamma-inducible protein 10, or an immunological portion thereof.

Such cells can be removed from the subject, transformed with an expression construct having a strong promoter for directing gene
5 expression and which encodes the interferon gamma-inducible protein 10, or the immunological portion thereof and once a sufficient level of expression is detected, re-introduced into the subject from which they were derived. Therein, such cells will express and secrete the interferon gamma-inducible protein 10, or the immunological portion thereof.

10 In the alternative, the expression construct can be used to directly vaccinate the subject, as is further exemplified and described herein.

The expression construct used while implementing the invention can be a viral eukaryotic expression vector ad described above of a naked DNA construct suitable for DNA vaccination, such as, but not limited to,
15 pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, which are available from Invitrogen, pCI which is available from Promega, pBK-RSV and pBK-CMV which are available from Stratagene, pTRES which is available from Clontech, and their derivatives.

Thus, according to a presently preferred embodiment of the present invention generating in the subject cells expressing recombinant interferon gamma-inducible protein 10, or an immunological portion thereof, is effected by vaccinating the subject with an expression construct encoding
5 interferon gamma-inducible protein 10, or the immunological portion thereof.

As already mentioned herein above, the expression construct includes transcription control sequences of any suitable type compatible with eukaryotic gene expression. Strong and effective control sequences
10 are preferably of choice. These sequences can be from a mammalian or viral source. Examples include, but are not limited to, RSV control sequences, CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β -actin control sequences, myosin control sequences, all of which are potent and effective control sequences, capable
15 of efficiently directing gene expression in either a plurality of cell types of specific cell types (e.g., tissue specific promoters).

The *in vivo* level of expression of the interferon gamma-inducible protein 10, or the immunological portion thereof can be readily monitored using serum samples derived from the treated subject. Such serum

samples can be analyzed for the level of interferon gamma-inducible protein 10, or the immunological portion thereof using assays well known to the skilled artisan, including, but not limited to, enzyme linked immunosorbent assay (ELISA), immunoprecipitation, Western blots, slot
5 and dot blots, magnetic bead separation, solid support arrays, affinity columns and phage or bacterial display assays. These methods are well known in the art and as such no further description thereof is provided herein.

The amount of interferon gamma-inducible protein 10, or an
10 immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies can be determined experimentally for various diseases, using experimental procedures similar to those described in detail in the Examples section that follows and as is further described hereinunder with respect to the pharmaceutical compositions of
15 the present invention.

The amount of circulating anti-interferon gamma-inducible protein 10 antibodies required to prevent or treat a disease is determinable using similar experimental procedures.

A pharmaceutical formulation according to the present invention includes, as an active ingredient, an amount of interferon gamma-inducible protein 10, or an immunological portion thereof, or of an expression construct encoding said interferon gamma-inducible protein 10, or said immunological portion thereof, sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies, or the anti-interferon gamma-inducible protein 10 antibodies themselves in an amount sufficient to achieve a desired therapeutic effect in the subject. The active ingredients can be administered to an organism *per se*, or in a pharmaceutical composition where it is mixed with suitable carriers or excipients.

Thus, each of the pharmaceutical compositions of the invention comprises, a pharmaceutically acceptable carrier approved for medical or veterinary administration and, as an active ingredient, an amount of interferon gamma-inducible protein 10, an immunological portion thereof, or of an expression construct encoding said interferon gamma-inducible protein 10, or said immunological portion thereof sufficient to elicit sufficient anti-interferon gamma-inducible protein 10 antibodies, or the anti-interferon gamma-inducible protein 10 antibodies themselves in an

amount sufficient to achieve a desired therapeutic effect in the subject.

The pharmaceutical compositions of the invention can be used for

- (i) breaking-down an immunological tolerance to interferon gamma-inducible protein 10 in a subject; (ii) generating self specific
- 5 immunity to interferon gamma-inducible protein 10 in a subject; (iii) preventing or treating an autoimmune disease in which activity of interferon gamma-inducible protein 10 is pivotal, in a subject; (iv) restricting a polarization of myelin basic protein specific T cells into Th2 cells in a subject; (v) preventing or treating multiple sclerosis in a subject;
- 10 and for (vi) inducing protective immunity against multiple sclerosis in a subject.

As used herein a "pharmaceutical composition" refers to the active ingredients as described herein mixed with other chemical components such as physiologically suitable carriers and excipients. The purpose of a

15 pharmaceutical composition is to facilitate administration of a compound to an organism.

Hereinafter, the terms "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an

organism and does not abrogate the biological activity and properties of the administered compound.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active compounds into
5 preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the compounds of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological saline buffer. For
10 transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the active ingredients can be formulated readily by combining with pharmaceutically acceptable carriers well
15 known in the art. Such carriers enable the active ingredients to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the

mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions, which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of

gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients
5 may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of
10 tablets or lozenges formulated in conventional manner.

For administration by inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane,
15 trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated

containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The preparations described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion.

- 5 Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

- 10 Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters
15 such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents

which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before
5 use.

The preparation of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

The pharmaceutical compositions herein described may also
10 comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include, but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin and polymers such as polyethylene glycols.

Pharmaceutical compositions of the invention preferably include an
15 immunization adjuvant approved by a regulatory entity such as the FDA for medical and/or veterinary use.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More

specifically, a therapeutically effective amount means an amount of the active ingredient effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within
5 the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from animal models. Such information can be used to more accurately
10 determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in experimental animals, e.g., by determining the LD₅₀ (lethal dose causing death in 50 % of the tested animals). The data obtained from these animal
15 studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in

view of the patient's condition. (See e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active antibodies which are sufficient to
5 create immunological memory.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of the present invention may, if desired, be presented
10 in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accompanied by a
15 notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and

Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

- 5 Suitable conditions indicated on the label may include treatment of diseases in which interferon gamma-inducible protein 10 plays a pivotal role, such as autoimmune disease, including multiple sclerosis, rheumatoid arthritis and other autoimmune diseases accompanied by inflammation in which IP-10 has a pivot function.

- 10 In general, the present invention is practiced by directly (i.e., administration of anti-IP-10 antibodies) or indirectly (administration of IP-10 or a construct capable of in vivo expression of IP-10) introducing anti-interferon gamma-inducible protein 10 antibodies to the subject in an amount sufficient to treat or prevent a disease, syndrome or a
15 manifestation associated therewith.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the

present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook *et al.*, (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson *et al.*, "Recombinant DNA", Scientific American Books, New York; Birren *et al.* (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and

5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites *et al.* (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA

(1990); Marshak *et al.*, "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures

5 therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

MATERIALS AND EXPERIMENTAL PROCEDURES

Rats:

10 Female Lewis rats, approximately six weeks old were purchased from Harlan (Jerusalem, Israel) and maintained under SPF conditions in an animal facility.

Peptide antigens:

Myelin Basic Protein (MBP) p68-86, Y G S L P Q K S Q R S Q D

15 E N P V (SEQ ID NO:1), was synthesized on a MilliGen 9050 peptide synthesizer by standard 9-fluorenylmethoxycarbonyl chemistry and purified by high performance liquid chromatography. Structure was confirmed by amino acid analysis and mass spectroscopy. Only peptides that were greater than 95 % pure were used in subsequent experiments.

Immunizations and active disease induction:

Rats were immunized subcutaneously in the hind foot pads with 0.1 ml of MBP epitope 68-86 (p68-86) dissolved in PBS (1 mg/ml) and emulsified with an equal volume of CFA (incomplete Freund's adjuvant
 5 supplemented with 4 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra in oil (Difco laboratories, Inc., Detroit, MI). Rats were then monitored for clinical signs daily by an observer blind to the treatment protocol. EAE was scored as follows: 0, clinically normal; 1, flaccid tail; 2, hind limb paralysis; 3, total hind limb paralysis, accompanied by an
 10 apparent front limb paralysis; and 4, total hind limb and front limb paralysis.

Reverse transcriptase polymerase chain reaction (RT-PCR)

analysis: RT-PCR analysis was utilized on brain samples according to a protocol described elsewhere (94). IP-10 specific oligonucleotide
 15 primers were designed based on its published sequence (NCBI Accession Number: U22520) as follows: Rat IP-10 sense 5'-CATGAACCCAAGTGCTGCTGTCGT-3' (SEQ ID NO:2); and Rat IP-10 antisense 5'-TTACGGAGCTCTTTTAGACCTTCT-3' (SEQ ID

NO:3). The PCR product was then cloned and its sequence was verified as described below.

Cloning and sequencing of PCR products:

The PCR product described above was cloned into a pUC57/T
 5 vector (T-cloning Kit, Cat. No. K1212, MBI Fermentas, Lithuania) and transformed into E. Coli according to the manufacturer's protocol. Each clone was then sequenced (Sequenase Version 2, USB, Cleveland, Ohio) according to the manufacturer's protocol.

DNA vaccination:

10 DNA vaccination was performed as previously described (94). A sequenced PCR product of rat IP-10 was transferred into a pcDNA3 vector (Invitrogen, San Diego, CA). Large scale preparation of plasmid DNA was conducted using Mega prep (Qiagen Inc., Chatsworth, CA). Cardiotoxin (Sigma, St. Louis, MO) was injected into the tibialis anterior muscle of 4-6
 15 weeks old female Lewis rats (10 μ M per leg). One week following injection rats were injected with 100 μ g DNA in PBS. Four-five days after the first immunization one rat from the group previously subjected to IP-10 DNA vaccination was sacrificed and transcription of IP-10 was verified using RT-PCR on tibialis anterior muscle samples. Thereafter,

identical doses of naked DNA vaccines were given 3-5 times with intervals of 6-7 days between each injection.

Production and purification of recombinant IP-10:

PCR product was recloned into a PQE expression vector, expressed
 5 in E. Coli (Qaigen, Chatsworth, CA) and then purified by an
 NI-NTA-supper flow affinity purification of 6xHis proteins (Qaigen,
 Chatsworth, CA). After purification the purity of the recombinant IP-10
 was verified by gel electrophoresis. The recombinant protein sequence
 was verified (N -terminus).

10 ***Western blot analysis:***

The recombinant rat IP-10, produced as described above, and
 commercially available recombinant mouse IP-10 (Cytolab, Rehovot,
 Israel) were each subjected to Western blot analysis according to the
 protocol described in details elsewhere (112) with a minor modification of
 15 using a 12 % (rather than 8 %) running gel. IgG from IP-10 DNA
 vaccinated rats, or IgG from normal rat serum (final concentration of
 1:500 each) were used as primary antibodies. A goat anti-rat alkaline
 phosphates conjugated antibody (Sigma) was used a secondary antibody.

BCIP (Sigma, 0.15 mg/ml) and NBT (Sigma, 0.3 mg/ml) were then used as a substrate.

Evaluation of anti-IP-10 ligand antibody titer in sera of DNA vaccinated rats:

A direct ELISA assay has been utilized to determine the anti-IP-10 antibody titer in DNA vaccinated rats. The recombinant IP-10 was coated onto 96 well ELISA plates (Nunc, Denmark), at a concentration of 50 ng/well. Rat anti-sera, in serial dilutions of from 2^8 to 2^{30} were added to the wells of the ELISA plates. A goat anti-rat IgG alkaline phosphatase conjugated antibody (Sigma) was used as a secondary antibody. p-Nitrophenyl Phosphate (p-NPP) (Sigma) was used as a soluble alkaline phosphatase substrate. The results are shown as \log_2 antibody titer \pm SE.

CNBr Purification of anti-IP-10 specific antibodies:

Recombinant rat IP-10 (5 mg) was bound to a CNBr activated Sepharose column according to the manufactures instructions (Pharmacia biotech, Cat. No. 17-0820-01). Anti-IP-10 specific antibodies from sera (IgG fraction) of DNA vaccinated rats were loaded on the column and then eluted by an acidic elution buffer (glycine, pH 2.5). Isotype determination

of the purified antibody (ELISA) revealed that purified antibodies are mostly of the IgG2a Isotype.

Cytokine determination in cultured primary spleen cells:

Spleen cells from EAE donor rats were stimulated *in vivo* (10^7 cells/ml) in 24 well plates (Nunc) with 100 μ M p68-86. After 72 hours of stimulation, supernatants were assayed for the protein level of various cytokines using semi-ELISA kits:

1. IFN- γ : rabbit anti-rat IFN- γ polyclonal antibody (CY-048, Innogenetics, Belgium) as a capture antibody, biotinylated mouse anti-rat monoclonal antibody (CY-106 clone BD-1, Innogenetics) as a detection antibody, and alkaline phosphatase-streptavidin (Cat No. 43-4322, Zymed, SF, CA) with rat recombinant IFN- γ as a standard (Cat. No. 3281SA, Life Technologies).

2. TNF- α : commercial semi-ELISA kit for the detection of rat TNF- α , (Cat. No. 80-3807-00, Genzyme, Cambridge, MA).

3. IL-4: mouse anti-rat IL-4 monoclonal antibody (24050D OX-81, PharMingen, San Diego, CA) as a capture antibody, and rabbit anti-rat IL-4 biotin-conjugated polyclonal antibody (2411-2D,

PharMingen) as a secondary antibody. Recombinant rat IL-4, purchased from R&D (504-RL), was used as a standard.

4. IL-10: commercial semi-ELISA kits for the detection of rat IL-10 (PharMingen, San Diego, CA).

FACS analysis:

FACS analysis was conducted according to the basic protocol described in details elsewhere (91). Intracellular staining of IFN- γ and IL-4 was done using a commercially available kit (LEUCOPERM, Serotec, Oxford, UK, Cat. No. BUF9) according to the manufacturer's protocol. A FITC labeled mouse anti-rat IFN- γ monoclonal antibody (Biosource, Nivelles, Belgium) and PE labeled mouse anti-rat IL-4 monoclonal antibody (Biosource) were used for direct staining. Cells were analyzed using a FACSCalibur (Becton Dickinson, Mountain View, CA). Data were collected for 10,000 events and analyzed using a Cell Quest program (Becton Dickinson).

Histopathology:

Histological examinations of hematoxylin and eosin-stained, formalin-fixed, paraffin-embedded sections of the lower thoracic and

lumbar regions of the spinal cord were performed. Each section was evaluated without prior knowledge of the treatment status of the animal.

The following scale was used: 0, no mononuclear cell infiltration; 1, 1 to 5 perivascular lesions per section with minimal parenchymal infiltration; 2, 5 to 10 perivascular lesions per section with parenchymal infiltration; and 3, over 10 perivascular lesions per section with extensive parenchymal infiltration. The mean histological score \pm SE was calculated for each treatment group.

Statistical analysis significance of differences was applied using the Student's t-test. A value of $p < 0.05$ was considered significant. Mann-Whitney sum of ranks test was used to evaluate significance of differences in mean of maximal clinical score. Value of $p < 0.05$ was considered significant.

EXPERIMENTAL RESULTS

IP-10 encoding DNA vaccine redirect antigen specific T cell polarization and suppresses EAE:

Cloned PCR products of rat IP-10 were ligated into a pcDNA3 eukaryotic expression vector and used as constructs for naked DNA vaccination. Rats were subjected to four weekly injections of the above

construct. Control rats were injected either with the pcDNA3 vector alone, pcDNA3- β -actin encoding construct or with PBS. Two months after the last immunization all rats were immunized with p68-86/CFA to induce active EAE. All control groups developed active disease that persisted for 5-6 days (Figure 1A, 6/6 in each group with a maximum clinical score 2.83 ± 0.18 , 2.5 ± 0.23 and 2.33 ± 0.23 in PBS, pcDNA3- β -actin and pcDNA3 immunized rats, respectively). In contrast, rats injected with the IP-10 naked DNA vaccine developed a markedly reduced degree of disease (Figure 1 incidence of 6/6 with a maximum clinical score of 1.16 ± 0.18 , $p < 0.016$ for the comparison of this treatment to PBS treated rats and $p < 0.02$ for the comparison with each of the other control groups). Disease suppression was accompanied by a marked reduction in perivascular mononuclear cell infiltration, as was histologically observed in a double blind evaluation (Table 3, $p < 0.001$).

Table 3
Targeted DNA vaccines encoding IP-10 markedly decrease CNS
mononuclear cell infiltration.

Treatment		Induction of EAE	Mean Histological Score
A	PBS	-	0±0
B	PBS	+	2.66±0.3
C	PcDNA3 alone	+	2.33±0.3
D	PcDNA3-IP-10	+	0.66±0.3*

5 * p<0.001 for D compared with either B or C

10 Table 3: At the peak of disease described above (Figure 1A) 3 rats per group were sacrificed and spinal cord samples were subjected to histological evaluation. The following scale was used: 0, no mononuclear cell infiltration; 1, 1 to 5 perivascular lesions per section with minimal parenchymal infiltration; 2, 5 to 10 perivascular lesions per section with parenchymal infiltration; and 3 >10 perivascular lesions per section with extensive parenchymal infiltration. The mean histological score of 18 different sections ± SE was calculated for each treatment group.

Thus, the autoimmune response subsequent to administration of the
 15 IP-10 encoding naked DNA vaccine leads to a marked reduction in mononuclear cell infiltration to the CNS and suppresses EAE (Figures 1A-D and Table 3).

To determine whether IP-10 encoding DNA vaccine altered antigen specific T cell polarization, spleen T cells derived from the rats were

cultured with MBPp68-86 and levels of Th1 Vs Th2 cytokines were determined. Rats administered with IP-10 encoding DNA vaccine showed a remarkable elevation in the *ex vivo* production IL-4 (Figure 1C, $p < 0.0001$ compared to each control group) together with a marked reduction in TNF- α production (Figure 1B, $p < 0.0001$ compared to control EAE rats and $p < 0.05$ compared to pcDNA3 treated EAE rats) and a significant reduction in IFN- γ production (Figure 1D, $p < 0.01$ to all control groups). Based on these findings was hypothesized that a DNA vaccine encoding IP-10 leads to *in vivo* neutralization of IP-10 and thus alters T cell polarization into high IL-4, low IFN- γ , low TNF- α producing T cells. To explore this hypothesis several consecutive experiments were conducted as described bellow.

IP-10 encoding DNA vaccine induces breakdown of tolerance to its gene product and generates immunity to native IP-10:

DNA vaccination can potentially elicit both cellular and humoral responses against products of a given construct. To determine whether the administration of IP-10 DNA vaccine leads to a breakdown of tolerance to self IP-10, Lewis rats were subjected to four weekly injections of the IP-10 DNA construct as described in Figures 2A-C. Two months after the last

immunization, when IP-10 specific antibody titer retained a baseline level (log₂ antibody titer of 7-8 in both DNA vaccinated and control untreated rats), these rats, and rats treated with either the pcDNA3 alone or PBS, were injected with p68-86/CFA to induce active EAE. Representative rats from each group were injected with CFA alone. At different time points blood serum and spinal cord fluid (SCF) from representative rats were analyzed for the presence of antibodies to IP-10, first by Western blot analysis (Figure 2A) and then by measuring log₂ of antibody titer. SDS-PAGE under reducing conditions followed by Western blot analysis revealed that both, these antibodies obtained from IP-10 DNA vaccinated rats bound a single 10 kDa fragment in supernatant of activated primary spleen T cells, and also a 8.7 kDa commercially available mouse IP-10 fragment (Figure 2A). The sequence of the 10 kDa native rat IP-10 has been verified (N-terminus analysis), as described elsewhere (105). Thus, self specific antibodies generated in IP-10 DNA vaccinated rats bind the natural form of self IP-10. An elicited titer to the above gene product was observed both in blood serum and SCF of IP-10 DNA vaccinated rats eight days after disease induction. This titer reached its maximal level on day 13 (Figure 2B, log₂ antibody titer of 22±1.2 and 24±2.6 in blood serum

and SCF of IP-10 DNA vaccinated rats compared with 10 ± 0.3 and 11 ± 0.3 in blood serum and SCF of pcDNA3 vaccinated rats and with 7 ± 0.2 in PBS treated EAE rats, $p < 0.001$ for the comparison of each control group with the respective IP-10 DNA vaccinated group). Taken together these results suggest that IP-10 naked DNA vaccination leads to a breakdown of immunological tolerance, resulting in the generation of an immunological memory which is turned on upon EAE induction (Figure 2B). CFA immunization alone did significantly elicit the production of self specific antibodies to IP-10 in DNA vaccinated rats (Figure 2C, in blood serum \log_2 antibody titer of 13 ± 0.5 compared with 7 ± 0 without CFA immunization, $p < 0.01$), though to a much lesser extent than an induction of active disease did (Figure 2C). Similar results were also previously obtained when tolerance to self was broken using MIP-1 α , MCP-1, TNF- α or FasL encoding DNA vaccines (90, 91, 93, 94, 96). The immunological basis of these differences is not fully understood yet.

IP-10-specific antibodies generated in DNA vaccinated EAE rats are neutralizing antibodies that inhibit both migratory properties and polarization of activated T cells:

Since DNA vaccination can potentially elicit both cellular and humoral responses against products of a given construct, it is difficult to know which of these responses contributed more to the development of EAE resistance in IP-10 DNA vaccinated rats (Figures 1A-D). The possibility that self-specific antibodies generated in DNA vaccinated rats against IP-10 contribute to the tolerant state in IP-10 DNA vaccinated rats was thus explored. At first, the *in vivo* competence of these antibodies to inhibit the IP-10 induced migration of a MBP specific encephalitogenic CD4⁺ T cell line was determined in a Boyden chamber assay (Figure 3A). Not only could these antibodies (CNBr purified) significantly inhibit encephalitogenic CD4⁺ T cell migration induced by recombinant rat IP-10, they could also prevent rat T cell migration induced by commercially (Cytolab, Rehovot, Israel) available 8.7 kDa fragment of the peptide (Figure 3A, $p < 0.001$). These antibodies had no effect on MCP-1 induced migration of these cells (data not shown). Thus the inhibitory effect of IP-10 self specific antibodies is chemokine specific. The addition of these

antibodies to MBP specific (p68-86) cultured spleen cells led to a significant decrease in TNF- α and IFN- γ production (Figure 3B, 1240 ± 60 vs. 460 ± 20 pg/ml, $p < 0.001$, and 29.8 ± 1.9 vs. 18.1 ± 1.2 ng/ml, $p < 0.01$ respectively). This reduction was accompanied by a marked increase in IL-4 production (Figure 3B, 48 ± 7 vs. 294 ± 12 pg/ml, $p < 0.0001$). Moreover, intracellular flow cytometry analysis (Figure 3C) clearly showed an apparent shift from high IFN- γ , low IL-4 producing CD4+ T cells (34 % high IFN- γ , low IL-4, 5 % high IL-4, low IFN- γ) in cultures that were not supplemented with anti-IP-10 antibodies, to low IFN- γ , high IL-4 producing CD4+ T cells in the presence of these antibodies (8 % high IFN- γ , low IL-4, 26 % high IL-4, low IFN).

Taken together, the data shows that self specific antibodies to IP-10 affect both the migratory properties and the cytokine profile of autoreactive T cells. Differential counting (by FACS) of macrophages (ED1 positive), CD4+ and CD8+ cells ruled out the possibility that these antibodies are depleting antibodies (not shown).

*Administration of self specific antibodies to IP-10 redirects
antigen specific T cell polarization towards Th2 and suppresses EAE:*

The *in vivo* properties of the self-specific anti-IP-10 antibodies may suggest that these antibodies may affect the *in vivo* function of autoimmune T cells and thus the regulation of EAE. The subsequent administration of these antibodies starting five days before the onset of disease led to a marked reduction in the clinical (Figure 4A) and histological scores of disease. That is, while all control EAE rats immunized with p68-86/CFA and then treated with either PBS, or with IgG (protein G purified) from either naive or pcDN3 treated EAE rats, developed severe EAE (Figure 4A, Mean Maximal score 3.83 ± 0.25 , 3.3 ± 0.8 and 3 ± 0.3) those treated with purified anti-IP-10 specific antibodies exhibited a mild form of the disease (mean maximal score of 1.16 ± 0.23 , $p < 0.001$ compared to each control group). Disease inhibition was accompanied by a significant, but not total, reduction in parenchymal mononuclear cell infiltration (mean histological score of 0.83 ± 0.16 vs 2.6 ± 0.2 and 2.3 ± 0.3 and 2.16 ± 0.3 in control groups, respectively). To assess the possibility that the administration of anti-IP-10 specific antibodies affected antigen specific T cell polarization, primary spleen T

cells from EAE rats treated with either PBS, normal rat IgG or anti-IP-10 antibodies were cultured with, or without, the MBP encephalitogenic determinant and supernatant levels of TNF- α (Figure 4B), IL-4 (Figure 4C) and IFN- γ (Figure 4D) were determined. The addition of anti-IP-10

5 antibodies to MBP specific (p68-86) cultured spleen cells led to a significant decrease in TNF- α and IFN- γ production ($p < 0.001$). This reduction was accompanied by a marked increase in IL-4 production ($p < 0.0001$). To further assess the possibility that neutralizing IP-10 shifts the T cell subset balance towards Th2 CD4+, primary T cells from these

10 cultured T cells were subjected to intra-cellular staining of IFN- γ and IL-4. About 60 % of control CD4+T cells (from rats treated with normal IgG) included high IFN- γ producing Th1 (32 %) and Th0 (27 %) cells and only 18 % of high IL-4 low IFN- γ producing "Th2" cells (Figure 5A). In contrast, primary T cells (CD4+) from MBP activated spleen T cells from

15 rats treated with anti-IP-10 antibodies exhibited a significant shift towards Th2 (Figure 5B, 44 % high IL-4, low IFN- γ producing T cells and only 6 % high IFN- γ , low IL-4 producing cells).

IP-10 encoding DNA vaccines interferes in the regulation of established EAE:

Finally, whether IP-10 encoding DNA vaccines may interfere in the regulation of established EAE was determined. Thus Lewis rats were immunized with MBPp68-86/CFA to induce active EAE, five, six and seven days later injected with either IP-10 or soluble β -actin encoding DNA vaccines (300 μ g/rat per injection) and monitored for the development and progression of disease by an observer blind to the experimental procedure (Figure 6A). While control and β -actin DNA vaccinated rats developed a severe manifestation of disease (mean maximal score 3.5 ± 0.23 and 3.3 ± 0.23 respectively) those treated with IP-10 encoding DNA developed significantly lower form of disease (mean maximal score 1.5 ± 0.66 , $p < 0.01$ compared with each control group) and went into fast remission. On day 16 blood sera from these rats were analyzed for IP-10 or β -actin specific antibody titer (IgG). In accordance with the results summarized in Figures 6A-B, control rats with developing EAE developed a significant ($p < 0.05$) antibody titer to IP-10 during the course of disease that was amplified ($p < 0.001$) following DNA vaccination (Figure 6B). EAE rats did not mount an increased antibody

titer to self β -actin and this titer did not accelerate within few days after rats were subjected to β -actin encoding DNA vaccination (Figure 6B). This further suggest that naked DNA vaccination encoding proinflammatory mediators augments a pre-existing response, that plays a function in the regulation of the autoimmune condition. These antibodies were neutralizing *in vivo* and could suppress EAE in adoptive transfer experiments.

Since in the Lewis rat model of MS the disease manifests a short acute form that persists for only 5-6 days, this model is not optimal for exploring the ability of IP-10 encoding DNA vaccines to treat a full blown disease. Other two commonly used models of MS in rodents are the SJL mice model for relapsing remitting MS, and the MOG p35-55 induced disease in C57/BL mice. Each of these models shares properties with human MS (116). Under the experimental conditions employed SJL mice develop a short form of disease that persisted for 5-7 days. Later on, 60-80 % of the recovered mice develop a short relapse. MOG induced disease persists for about 30 days (Figure 7). This makes this model more practical for studying a full blown disease. C57/BL mice were subjected to active induction of EAE. On day 15 these mice were separated into four

groups of equally sick mice (six mice per group) and subjected to a repeated administration (3 times, days 15, 16, 17) of either IP-10 or soluble β -actin encoding DNA vaccines, empty vector (100 μ g each) or PBS. Only those administered with the IP-10 encoding DNA vaccine went

5 into a rapid remission (Figure 7, day 23, 0.66 ± 0.26 Vs 2.2 ± 0.3 , 2.3 ± 0.23 and 2.5 ± 0.3 respectively, $p < 0.01$). At this time (day 25) sera from 3 mice per group were analyzed for the development of anti IP-10 and anti β -actin antibodies as described in with respect to Figures 6A-B. Mice administered with IP-10 encoding DNA vaccine developed a significantly

10 elevated antibody titer to IP-10 (log₂ Ab titer of 22 ± 1 Vs 13 ± 0.5 in control EAE, $p < 0.001$, and 8 ± 0 in naive mice). This further implies that IP-10 encoding DNA vaccination can be used as a powerful tool to generate protective autoimmunity to IP-10 and thus treat ongoing MS.

15 It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of

a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with
5 specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art.

Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this

10 specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission

15 that such reference is available as prior art to the present invention.

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